JOURNAL OF BACTERIOLOGY, Sept. 1979, p. 770-774
0021-9193/79/09-0770/05$02.00/00

Vol. 139, No. 3

Escherichia coli Murein-DD-Endopeptidase Insensitive to β-Lactam Antibiotics

WOLFGANG KECK* AND ULI SCHWARZ

Max-Planck-Institut für Virusforschung, Abteilung Biochemie, D-7400 Tübingen, West Germany

Received for publication 6 July 1979

A novel endopeptidase degrading the peptide cross-links in sacculi has been isolated from *Escherichia coli* and purified to homogeneity. The enzyme has a molecular weight of 30,000 and, in contrast to already known enzymes of similar specificity, remains fully active in the presence of β-lactam antibiotics. In addition, it is exceptional in being inhibited by single-stranded deoxyribonucleic acid and by some polynucleotides. The possible role of the enzyme in cell division is discussed.

Hydrolytic enzymes (1, 2, 6, 11, 12, 18) participate in the metabolism of murein sacculi (21) and undoubtedly play a role in the maintenance of bacterial cell shape. In *Escherichia coli*, however, the precise biological role of the many murein hydrolases which have been identified remains largely obscure. Among these enzymes, endopeptidases split peptide bridges cross-linking the glycan chains of murein and are strongly inhibited by penicillins, although they are not assumed to be the lethal targets of β-lactam antibiotics (5, 9). During purification of a penicillin-sensitive DD-endopeptidase found to be identical with D-alanine-carboxypeptidase IB (18), we found a novel species of DD-endopeptidase which was totally insensitive to penicillin G; some properties of the enzyme have been described previously (20; U. Schwarz, W. Keck, S. Klencke, and H. Mett, Abstr. Symp. Functions Microb. Membranes, 1977). The enzyme thus belongs to a class of hydrolases which remain active in the presence of penicillin and may be the cause of penicillin-induced cell lysis.

In this paper we report on the purification to homogeneity and the properties of the novel endopeptidase. This enzyme is interesting not only because of its biological function but also because it has already been proven to be a powerful analytical tool; we have used it to elucidate the arrangement of the glycan chains within macromolecular sacculi (20).

MATERIALS AND METHODS

Cell extraction and initial enrichment of endopeptidase. The enzyme was isolated from *E. coli* PA 3092 (14) (*dpm lys*), harvested at mid-exponential growth phase, and kept frozen at −70°C. Cells (200 g, wet weight) were opened by shaking with glass beads in a cell mill in 500 ml of Tris-maleate (10 mM; pH 6.8)–10 mM $MgSO_4$, containing 40 μg of DNase per ml (from bovine pancreas ~450 Kunitz units per mg; Serva). The filtered suspension (4) was centrifuged (60 min at 48,000 × *g*), the supernatant was dialyzed three times against 5 liters of Tris-maleate buffer (10 mM; pH 5.2), 10 mM $MgSO_4$, and 0.1 mM dithioerythritol, cleared by centrifugation (30 min, 28,000 × *g*), and applied on a column (2.6 by 31 cm; flow rate, 32 ml/h) of CM Sepharose CL-6B (Pharmacia Fine Chemicals) equilibrated with dialysis buffer. The column was eluted with a concave gradient (380 ml of equilibration buffer, 320 ml of 0.5 M KCl in the same buffer). Endopeptidase was found in a broad peak between 0.1 and 0.3 M KCl. The pooled fractions with endopeptidase were dialyzed three times against 5 liters of Tris-maleate buffer (10 mM, pH 8.0, 0.1 mM dithioerythritol). The dialysate was centrifuged (30 min at 28,000 × *g*) and further fractionated on DEAE-Sepharose CL-6B (column, 2.6 by 40 cm; flow rate, 33 ml/h; Pharmacia) with buffer as used for dialysis. Neither endopeptidase nor transglycosylase was retained on the column; both were eluted with 80% recovery in the flow-through. The enzyme pool was concentrated (Amicon, P10 membrane) to a volume of 28 ml, dialyzed two times against 2 liters of buffer (10 mM potassium phosphate, pH 7, 0.1 mM dithioerythritol), and used for final purification on polyuridylic acid [poly(U)]-Sepharose (Pharmacia).

Enzyme assays. (i) Total murein hydrolase activity. Total murein hydrolase activity was measured with sacculi as a substrate (3); the sacculi were labeled with DL-*meso*-[^{3}H]diaminopimelic acid (specific activity, 19 Ci/mmol; Service des Molécules Marquées, Gif-sur-Yvette, France). Murein hydrolase action results in a liberation of low-molecular-weight reaction products which is the basis of our enzyme test (3). Normally, 10 μl of enzyme solution to be tested was incubated for 60 min at 37°C in the following test mixture: 10 μl of sacculi suspension (0.5 mg of murein per ml; 1.5 × 10^6 cpm/ml); 50 μl of Tris-maleate buffer (20 mM; pH 6.0; 20 mM $MgSO_4$; 0.4% Triton X-100); and water up to a total volume of 100 μl. Undigested murein was precipitated by the addition of 100 μl of 1% *N*-cetyl-*N*,*N*,*N*-trimethylammoniumbromide. After centrifugation (2 min at 12,000 × *g*), 100 μl of

supernatant was counted in 2 ml of toluene Triton X-100 scintillation cocktail.

(ii) Endopeptidase activity. Endopeptidase activity was determined with the dimeric muropeptide C3 as a substrate which is converted into a monomeric reaction product (3). Standard conditions for the assay of endopeptidase activity were as follows. In a 40-μl total volume (10 mM Tris-maleate, pH 6.0, 10 mM $MgSO_4$, 0.2% Triton X-100), 5 μl of substrate, ([^{3}H]-diaminopimelic acid [Dpm] muropeptide C3 [13]; final concentration, 2 μM; 2.3 × 10^3 cpm/80 pmol) was incubated with 2 μl of enzyme solution. After incubation for 8 min at 37°C, the test mixture was boiled for 3 min and directly applied onto paper chromatograms as described (13). The amount of protein was measured by the method of Lowry et al. (7). The specific activity was calculated as picomoles of C3 digested per milligram of protein per minute.

The action of endopeptidase on cross-links in intact sacculi was assayed as mentioned previously (10).

(iii) Transglycosylase. Transglycosylase liberates low-molecular-weight reaction products from sacculi and was used as a measure of enzyme activity as indicated (4).

(iv) D-Alanine carboxypeptidase. D-Alanine carboxypeptidase liberates D-alanine from UDP-muramyl-pentapeptide (6). The assay conditions were the same as used previously (2, 6). Our substrate, UDP-*N*-acetyl-muramyl-L-Ala-D-Glu-*meso*-Dpm-D-Ala-^{14}C-D-Ala (2 × 10^4 cpm/nmol), was a kind gift from D. Mirelman.

RESULTS AND DISCUSSION

Purification of the endopeptidase. A serious problem for the final purification of the enzyme was the formation of a stable complex with another murein hydrolase, murein transglycosylase (4). The stability of the complex is remarkable; it remains intact during gel permea-

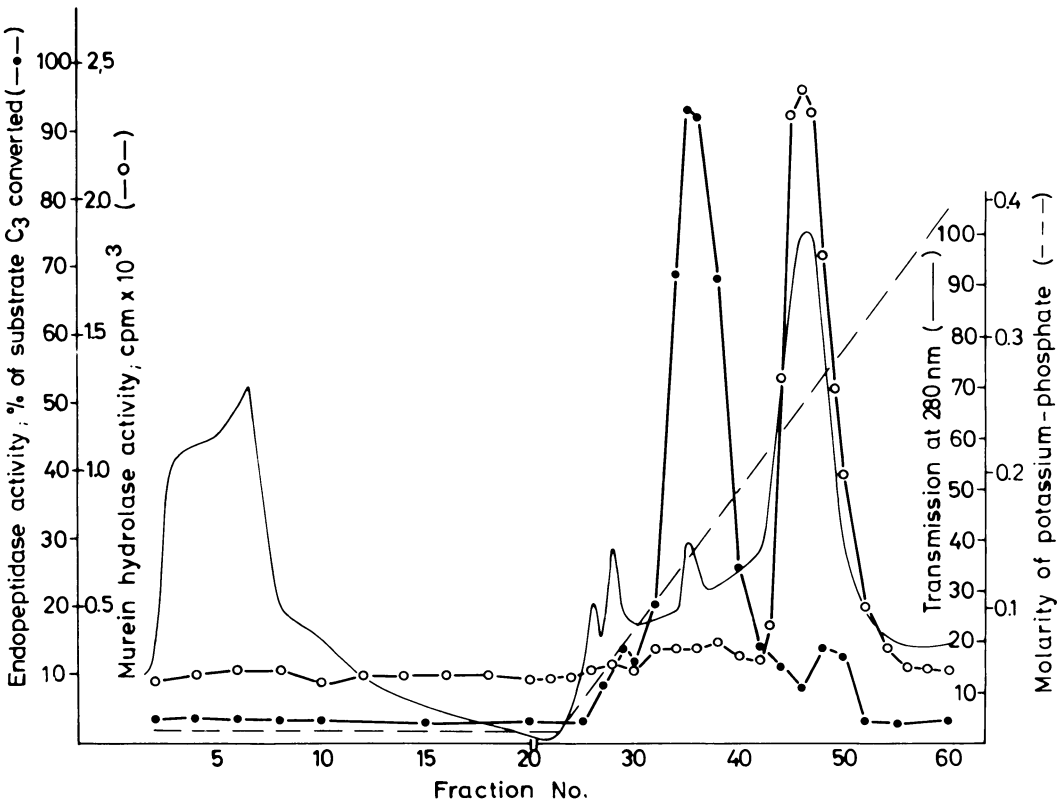

FIG. 1. *Separation of endopeptidase from transglycosylase by affinity chromatography on poly(U)-Sepharose 4B. The enzyme concentrate obtained after chromatography on DEAE-Sepharose (see text) was applied on a poly(U)-Sepharose 4B (Pharmacia) column (0.6 by 8 cm; 10 ml/h), equilibrated with 10 mM potassium phosphate buffer, pH 7.0, with 0.1 mM dithioerythritol. A linear gradient (100 ml of potassium phosphate, 10 mM, pH 7.0, 0.1 mM dithioerythritol, 100 ml of the same buffer at a 1 M concentration) was used for elution. Enzyme-containing peak fractions (fractions 1 through 20 were collected at 30 min, and the following fractions were collected at 15-min intervals) were dialyzed (10 mM potassium phosphate, pH 7.0, 0.1 mM dithioerythritol) and used for a characterization of the endopeptidase (Table 1).*

tion and ion-exchange chromatography, both enzyme activities being eluted together in a sharp single peak (data not shown).

Another unusual feature of the endopeptidase gives the key to its final purification. The enzyme interacts very strongly with nucleic acids and nucleic acid analogs, and the interaction is paralleled by inactivation of the enzyme (see below). Based on this observation, the endopeptidase was finally separated from transglycosylase by affinity chromatography on poly(U)-Sepharose. Figure 1 shows the complete separation of the two enzyme species. Endopeptidase activity in the highly purified fraction was enriched approximately 20,000-fold as compared with the crude cell extract (Table 1). The very high degree of enrichment may be explained by the presence in the crude cell extracts of small amounts of inhibitory oligonucleotides which may have persisted even after DNase treatment.

FIG. 2. *Purity and molecular weight of the endopeptidase. Photograph from a 10% sodium dodecyl sulfate-polyacrylamide slab gel (8) on which endopeptidase (from fraction 35, Fig. 1) and marker proteins had been separated by electrophoresis: (A) endopeptidase (molecular weight 30,000); (B) chymotrypsinogen (molecular weight 25,000); (C) ovalbumin (molecular weight 45,000); (D) bovine serum albumin (molecular weight 68,000); (E) RNA polymerase (molecular weight 39,000, 155,000, and 165,000).*

Properties of the purified endopeptidase. Our enzyme preparation yields a single band with an apparent molecular weight of 30,000 upon sodium dodecyl sulfate gel electrophoresis (Fig. 2). The purified enzyme, the activity of which is stimulated by Triton X-100, was used for determination of pH optimum, activation by ions, the isoelectric point, the Michaelis constant, and sensitivity to β-lactam antibiotics. Endopeptidase was assayed as follows. The test mixture was incubated with 0.03 μg of protein of the final purification step (Table 1). For measuring the V_{max} (0.41 pmol/mg of protein per min) and K_m (4×10^{-6} M), substrate at concentrations between 1.6 and 40 μM was used, and the values were calculated from a plot of the data based on a Lineweaver-Burk plot after regression analysis of the data (regression coefficient, 0.87). For determination of the pH optimum (6.0), 10 mM Tris-maleate buffer at pH between 5.2 and 8.6 was used. Mg^{2+} dependence of endopeptidase activity was measured in the molarity range from 0 to 100 mM $MgSO_4$; 5 mM was optimal. Addition of 0.1 to 1% Triton X-100 to the enzyme increased its activity by a factor of 2. The isoelectric point of the endopeptidase (6.8) was determined by flatbed electrofocusing (LKB Instrument GmbH.) in a granulated gel (pH range, 2 to 11; constant voltage, 200 V/14 h, 400 V/3 h, 800 V/3 h). The enzyme is distinct

TABLE 1. *Purification of endopeptidase*[a]

Purification step	Total vol (ml) of fractions	Total protein (mg)	Sp act	Yield (%)	Purification factor
Crude extract	500	7,250	5.4	100	1
CM-Sepharose column	425	413	118	125	22
DEAE-Sepharose column after concentration	28	1.96	10,800	54	2,000
Poly(U)-Sepharose	12.5	0.164	113,000	47	20,900

[a] Enzyme purification by chromatography on CM-Sepharose and on DEAE-Sepharose is described in the text. The procedure of chromatography on poly(U)-Sepharose is outlined in the legend to Fig. 1. Enzyme activities were tested as described in the text. One unit of enzyme activity is equivalent to 1 pmol of substrate degraded per mg of protein per min at 37°C.

from D-alanine-carboxypeptidase IB, which also shows endopeptidase activity (18), in that the new endopeptidase is fully active in the presence of penicillin G or of other β-lactam antibiotics at 100 μg/ml, including ampicillin, cephaloridine, cephalexin, mecillinam, cefoxitin, and piperacillin. Carboxypeptidase IB is highly sensitive to penicillin G (18). Furthermore, our enzyme operates exclusively as an endopeptidase; we could not detect any DD-carboxypeptidase activity with UDP-muramyl-pentapeptide as a substrate.

The inhibition of the endopeptidase by DNA and by polynucleotides is striking (Fig. 3). Single-stranded polymers are much more effective than double-stranded structures. Double-stranded DNA from calf thymus and from beef lymphocytes are almost ineffective, whereas the same compounds upon dissociation into single strands are strongly inhibitory (Fig. 3B). Single-stranded structure also is important for the inhibitory effects of polynucleotides. Polyinosinic acid-polycytidylic acid (double stranded) and polyadenylic acid [poly (A)] are very ineffective inhibitors; poly(A) forms double strands at the pH used in our experiments (15). In contrast, the single-stranded polyguanylic acid and poly(U) are strong inhibitors (Fig. 3A).

It is too early to speculate on the biological relevance of the inhibition of the endopeptidase by DNA. Although isolation of proper mutants may shed light on the biological role of the enzyme, we see no way to prove or disprove whether DNA may serve as a modulator of enzyme activity. Furthermore, dramatic inhibition by nucleic acids and by oligonucleotides may be the result of a more general phenomenon rather than a specific property of the endopep-

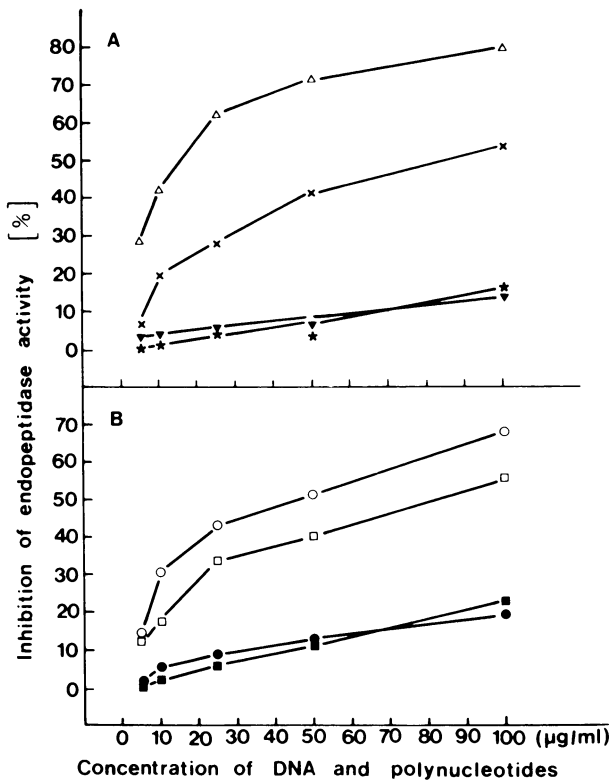

FIG. 3. *Inhibition of endopeptidase by DNA and by various polynucleotides. The enzyme used in the test mixture was purified as described and then stabilized by addition of bovine serum albumin (170 μg/ml of enzyme solution). Endopeptidase was assayed essentially as described in the text. In a 40-μl total volume (10 mM Tris-maleate, pH 6.0, 10 mM $MgSO_4$, 0.2% Triton X-100), 5 μl of substrate ([^{3}H]Dpm muropeptide C3; final concentration, 8.75 μM; 6.8 × 10^4 cpm/350 pmol) was incubated with 10 μl of enzyme solution for 30 min at 37°C. (A) Poly(U) (△); polyguanylic acid (×); polyinosinic acid-polycytidylic acid (*); poly(A) (▼). (B) DNA from calf thymus, double stranded (●) and single stranded (○); DNA from beef lymphocytes, double stranded (■) and single stranded (□).*

tidase; murein transglycosylase activity is also reduced 95% by 3 μg of single-stranded DNA per ml (W. Kusser and U. Schwarz, Eur. J. Biochem., in press).

The existence of a novel endopeptidase in *E. coli* was not quite unexpected. In early (12) and also later reports (3, 9, 11, 17, 18), a multiple set of endopeptidases with graded penicillin sensitivities had been implicated. Also recently, a report on a partial enrichment of a penicillin-insensitive DD-endopeptidase from *E. coli* has appeared (19). A comparison between this enzyme and ours, however, must await more sufficient data.

Our considerations on the possible biological role of the penicillin-insensitive endopeptidase originate from the fact that the enzyme accepts the intact murein sacculus as a substrate, resulting in degradation of the murein network into glycan chains. Because the glycan chains in the sacculus show an orientation parallel to the plane of cell division (20), and because during normal septum formation the sacculus is enzymatically degraded in the division plane (16), one may assume that the novel endopeptidase plays an essential role in the complex process of cell division. Further support for this speculation comes from the observation that a topologically controlled hydrolysis of the sacculus at the division site takes place in the presence of even high concentrations of penicillin (16); the enzyme involved consequently must be penicillin insensitive, as is the case for our endopeptidase.

ACKNOWLEDGMENTS

We thank I. Baxivanelis, A. Funk, V. Kastner, and R. Wutz for technical help.

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